



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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6/15/90

In re application of :
MARCUS A. HORWITZ : Group Art Unit: 183
: Examiner: A. MOHAMED
Serial No.: 232,664 :
Filed: August 16, 1988 :
For: LEGIONELLOSIS VACCINES :
AND METHODS FOR THEIR :
PRODUCTION : Docket No.: 70-155

DECLARATION UNDER RULE 132

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Tim Conley
May 30 1990

Sir:

I, Marcus A. Horwitz, M.D., declare and state that:

1. I am the inventor in the above-identified patent application.

2. I received my M. D. Degree from Columbia University College of Physicians and Surgeons, New York, New York in June of 1972 and am currently Professor of Medicine and of Microbiology and Immunology, Chief, Division of Infectious Diseases, Department of Medicine, UCLA School of Medicine, Center for the Health Sciences at Los Angeles, California. A copy of my current Curriculum Vitae detailing my Education, Internship and Residency, Public Health Service Positions, Clinical Fellowships, Research Fellowships, Faculty Positions, Certifications, Affiliations, Honors and Awards, Scientific and Editorial Boards and Study Sections, Publications, Abstracts, and Presentations at National or International Meetings

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is attached hereto as Exhibit 1. As indicated in my Curriculum Vitae, I have extensive experience in the fields of Microbiology, Immunology and Infectious Diseases.

3. I have reviewed the Official Action dated March 5, 1990, and have carefully studied the Examiner's statements therein and all of the references cited by the Examiner.

4. Initially, I understand that the Examiner has objected to a number of the terms and terminology used in the claims of my invention as being "indefinite and confusing". With all due respect to the Examiner, these are commonly used terms that anyone skilled in the fields of Microbiology, Immunology or Infectious Diseases understands. Additionally, I also discussed these various terms at length in the specification of my patent application.

5. More specifically, "intracellular pathogen" is a well recognized term in the medical fields of Immunology and Infectious Diseases and is discussed in detail in my patent application at pages 1-6. In fact, as evidenced by my Curriculum Vitae, as early as 1980 I had been making numerous presentations at national and international meetings regarding intracellular pathogens and parasites and intracellular infection. The same is true for the term "extracellular product" which is widely recognized and clearly understood by those skilled in the fields of Molecular Biology, Immunology, and Infectious Diseases and is discussed in my patent application beginning at page 6. Additionally, "adjuvant compound" is a well recognized term in the field of Immunology, and, in fact, refers to a very limited class of compounds which enhance the immune response of a given vaccine. Exemplary "adjuvant compounds" are discussed in my application and the few remaining members in this class of compounds are well known by my peers. Similarly, known cleavage methodologies such as the cyanogen bromide technique discussed in my application (page 8, lines 17-25) produce easily

determined "smaller subunits", a well recognized term in the field of Microbiology.

6. My reading of the Official Action indicates that the Examiner seems to be saying that my invention is limited to treating Legionnaire's Disease. This is not, in fact, the case as my invention is properly applicable to the entire class of intracellular pathogens which produce extracellular products that stimulate a strong lymphocyte proliferative response. Considering that my invention is a process for producing vaccines as well as the vaccines so produced, there appears to be no doubt that my invention will operate with respect to all intracellular pathogens for which it is possible to identify an extracellular product which stimulates a strong lymphocyte proliferative response.

7. To further verify the propriety of the claimed scope of my invention I conducted an additional set of experiments closely paralleling the Legionella pneumophila examples discussed in my application only directed against the causative agent of tuberculosis, Mycobacterium tuberculosis. As noted at page 1 of my application, intracellular pathogens are responsible for the estimated ten million new cases of tuberculosis per year.

8. As with my Legionella pneumophila experiment, in order to establish a foundation for studying cell-mediated immunity against M. tuberculosis, I first set up an animal model system of experimental tuberculosis. Under my direction and control, my assistants and I had an aerosol delivery device constructed in which guinea pigs could be infected by the respiratory route, which, as with L. pneumophila, is the most common way humans become infected with M. tuberculosis.

9. These initial pilot studies investigated the relationship between the aerosol dose of M. tuberculosis and the course of infection in the guinea pigs, as I wanted to determine an optimal aerosol dose to use in an experimental model of tuberculosis. Such

a dose should be large enough to cause clinical illness, but not so large as to result in severe morbidity. In my system, guinea pigs exhibited a clear-cut dose response to M. tuberculosis, i.e., as the aerosolized dose increased, so did the severity of illness, the number of macroscopic lung surface tubercles, and colony forming units (CFU) in the lung. A representative experiment is shown in Table I (Tables I - XII are attached hereto as Exhibit 2).

10. These studies suggested that the optimal aerosol concentration was approximately 10^4 bacilli/ml. Animals infected with this dose developed measurable clinical illness, i.e., mild weight loss and fever and had easily detectable and quantifiable numbers of lung surface tubercles.

11. Given a particular aerosol dose, I then wanted to determine the uniformity of infection with respect to the number of surface lesions and CFU in the lung. Table II reveals that aerosolization of a 10 ml suspension of M. tuberculosis in a concentration of 3×10^4 bacilli/ml resulted in a consistent number of macroscopic surface lesions and CFU in the lung within and between experiments.

12. Having established a system for reliably infecting guinea pigs with M. tuberculosis, my group and I then investigated to determine if infected guinea pigs develop cell-mediated immune responses to purified protein derivative (PPD), a protein complex that is able to elicit cell mediated immune responses in immune human subjects. My studies revealed that guinea pigs infected with M. tuberculosis exhibit a dose-dependent lymphocyte proliferative response to PPD (Table III) and develop cutaneous delayed-type hypersensitivity to PPD (Table IV).

13. To establish that extracellular products of M. tuberculosis stimulate cell-mediated immunity, my group and I assayed an ammonium sulfate precipitation fraction of these molecules for its capacity to stimulate splenic lymphocyte

proliferation and cutaneous delayed-type hypersensitivity in guinea pigs infected with M. tuberculosis.

14. To determine if extracellular products, in this case proteins, of M. tuberculosis are recognized by immune lymphocytes (lymphocytes from guinea pigs infected with M. tuberculosis), we studied the capacity of such lymphocytes to proliferate in response to an ammonium sulfate precipitation fraction of these proteins containing the major extracellular protein. The immune, but not control lymphocytes, proliferated strongly in response to these extracellular proteins.

15. To determine if extracellular proteins of M. tuberculosis stimulate cutaneous delayed-type hypersensitivity, we skin-tested guinea pigs infected with M. tuberculosis with an ammonium sulfate precipitation fraction of these proteins containing the major extracellular protein. Infected guinea pigs but not control uninfected guinea pigs exhibited marked erythema and induration in response to the ammonium sulfate precipitation fraction (Table VI).

16. These experiments showed that extracellular proteins in M. tuberculosis culture filtrates could stimulate cell-mediated immunity, the type of immunity that provides host defense against M. tuberculosis and other intracellular parasites.

17. To determine if partially purified major extracellular protein of M. tuberculosis (MEP-TB) stimulates cell-mediated immunity, my assistants and I partially purified the MEP-TB by molecular sieve chromatography and tested the capacity of immune lymphocytes to proliferate in response to it. On SDS-PAGE, the partially-purified MEP-TB had an apparent molecular weight of approximately 68 Kd and was the only protein visible on a coumassie stained gel. Lymphocytes from guinea pigs infected with M. tuberculosis but not from control uninfected guinea pigs proliferated in response to MEP-TB. The response of immune lymphocytes to MEP-TB was comparable to that of the ammonium

sulfate precipitation fraction tested earlier and comparable to or greater than that to PPD (Table VII).

18. To confirm further the capacity of MEP-TB to stimulate cell-mediated immunity, my assistants and I purified the molecule off of a Western blot and tested its capacity to stimulate immune lymphocytes. We subjected M. tuberculosis supernatant proteins to SDS-PAGE, transferred the proteins to nitrocellulose paper, cut the paper into small strips, eluted MEP-TB and other proteins off the strips and tested them in a lymphocyte proliferation assay. The eluted protein from the strip containing MEP-TB stimulated a strong lymphocyte proliferation response whereas molecules eluted off of all the other strips did not.

19. To determine if vaccination with extracellular proteins of M. tuberculosis induces a cell-mediated immune response, my assistants and I vaccinated guinea pigs subcutaneously with the ammonium sulfate precipitation fraction of these molecules and determined if the extracellular proteins could induce splenic lymphocyte proliferative and cutaneous delayed-type hypersensitivity responses. We found that guinea pigs immunized with the extracellular proteins, but not sham-immunized control guinea pigs, exhibited marked lymphocyte proliferation (Table VIII) and cutaneous delayed-type hypersensitivity (Table IX) to these proteins. Sites of skin responsiveness were biopsied and histology revealed an inflammatory cell infiltrate with a mononuclear cell predominance (greater than 70%), consistent with a classical delayed-type hypersensitivity response.

20. These studies showed that vaccination with extracellular proteins as taught in my patent application could induce cell-mediated immunity, the type of immunity important in host defense against M. tuberculosis and other intracellular pathogens. These studies confirmed the feasibility of using these extracellular products as a vaccine against this specific intracellular pathogen.

21. To further verify that vaccination of guinea pigs with partially purified MEP-TB could induce a cell-mediated immune response, my group and I vaccinated guinea pigs with MEP-TB partially purified by molecular sieve chromatography and tested the animals for cutaneous delayed-type hypersensitivity to MEP-TB. We immunized guinea pigs subcutaneously twice, three weeks apart, with 40 μ g of MEP-TB in mineral oil. Control guinea pigs were sham-immunized by injection with mineral oil only. The immunized but not control guinea pigs exhibited marked erythema at skin test sites.

22. To confirm that vaccination of guinea pigs with M. tuberculosis Extracellular Proteins induces protective immunity, my group and I immunized guinea pigs with the extracellular proteins and challenged them with an aerosol of M. tuberculosis. Six guinea pigs were immunized twice, three weeks apart, with 120 μ g of Extracellular Proteins in mineral oil (80% light mineral oil; 20% mannide monooleate). Six control guinea pigs were sham-immunized by two injections each with the mineral oil preparation only. The animals were challenged with an aerosol generated from a 10 ml suspension containing 5×10^3 colony forming units of M. tuberculosis, Erdman strain/ml. The animals were observed for illness and weight loss for nine weeks, sacrificed, and CFU of M. tuberculosis in their right lung and spleen were determined.

23. Control guinea pigs but not immunized guinea pigs exhibited failure to thrive by three weeks after challenge. The control animals began to look lethargic, and they exhibited poor weight gain in comparison to immunized animals (Table XI). The weight of control animals was markedly less than that of immunized animals throughout the remainder of the experiment (Table XI). At week nine, the control animals weighed an average of 122 grams less than immunized animals; a total body weight difference of 14% between the two groups.

24. At sacrifice, immunized animals had markedly fewer (1.1 log) CFU of M. tuberculosis in their lungs than control animals (log mean \pm SD 6.4 ± 0.6 CFU vs. 7.5 ± 1.2 CFU in the right lung). Immunized guinea pigs also had markedly fewer (1.2 log) CFU of M. tuberculosis in their spleen (log mean \pm SD 5.4 ± 0.4 CFU vs. 6.6 ± 1.2 CFU).

25. The level of protective immunity seen here in a guinea pig model of tuberculosis can be compared with that seen in my guinea pig model of Legionnaires' disease. In my previous study, guinea pigs immunized with the Major Secretory Protein of Legionella pneumophila (MEP-LP) had 1 to 1½ logs fewer bacteria on average in their right lungs than controls, similar to the difference in CFU of M. tuberculosis in the lungs of immunized and control guinea pigs. In the case of Legionnaires' disease, which unlike M. tuberculosis can be rapidly fatal, this difference in CFU represented a life or death difference. Almost all of the immunized guinea pigs survived whereas all the control guinea pigs died.

26. I believe that the foregoing clearly establishes the broad based applicability of my invention to the class of intracellular pathogens producing the appropriately reactive extracellular products as disclosed in my patent application. M. tuberculosis is markedly different from L. pneumophila, yet through evolutionary convergence these organisms share common mechanisms and pathways making them susceptible to the method and vaccines of my invention.

27. I understand that the Examiner also has objected to the term "effective amount" in my claims because it does not recite what is the actual effective amount. Again, with all due respect, the term "effective amount" is as specific as is possible in this contest. It has been my experience as one skilled in the fields of Molecular Biology, Immunology and Infectious Diseases that the

effective amount of a vaccine compound will vary between species and even between individuals of those species. Thus, while it is possible to extrapolate a reasonably anticipated effective amount of a new vaccine for human or mammalian vaccination purposes based upon the results of the laboratory animal studies disclosed in my application (see the discussion at page 24, lines 9-15 of my application), it is not within the skill of the art to do so accurately. As a result, a reasonable amount of experimentation is commonly required to establish the actual effective amount with reasonable certainty and consistency. Doing so on the basis of my disclosure is well within the skill of those who practice in the relevant medical fields.

28. I also understand that the Examiner has rejected my claimed invention as being anticipated by or obvious in view of United States Patent No. 4,780,407 issued to Strosberg et al. I have reviewed the Strosberg et al patent in detail and particularly note that the monoclonal antibodies discussed therein are not extracellular products of intracellular pathogens. Rather, monoclonal antibodies are extracellular products of host cells which in turn are hybrid cell lines produced through the fusion of immunized murine splenocytes and myeloma cells. Thus, monoclonal antibodies have nothing to do with intracellular pathogens.

29. Moreover, I also note that the Strosberg et al patent is primarily directed to methods for purifying monoclonal antibodies which can then be utilized to isolate and purify antigens for use in the preparation of vaccines. This is conventional vaccine production technology and is unrelated to the utilization of extracellular products of intracellular pathogens as vaccines.

30. Lastly, I also note that none of the other patents or articles cited by the Examiner discloses or suggests that extracellular products of intracellular pathogens can be utilized as vaccines. Rather, all of these references speak to traditional

vaccine technologies utilizing bacterial surface components. As discussed in my patent application at page 5, lines 18 - 20 such conventional vaccines may actually be contraindicated when dealing with intracellular pathogens as they may actually stimulate the proliferation of these infectious organisms rather than resolving the infection.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine, or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified patent application or any patent issuing thereon.

Date: 5-25-90

Marcus A. Horwitz
Marcus A. Horwitz, M.D.